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Starvation-Survival Patterns of Sixteen Freshly Isolated Open-Ocean Bacteria†

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Sixteen marine isolates from a NORPAX cruise, which were transferred once on medium after initial isolation, survived nutrient deprivation for at least 8 months (longest period test). All but one isolate remained cellularly intact, although their sizes and shapes changed greatly, and all became smaller, decreasing in size from 40 to 79%. Three starvation-survival patterns were demonstrated, namely (i) an initial increase in viable cells followed by a decrease until a constant number was reached, (ii) an increase in viable cells until a constant number was reached, and (iii) a decrease in viable cells until a constant number was reached. One isolate from each starvation-survival pattern was starved for 8 months and then was tested in comparison with 4-month-starved Ant-300 for [^{14}C]glutamic acid uptake, respiration, and incorporation. The response to glutamic acid was rapid and linear in each case. The data indicate that the starvation-survival of Ant-300 is not an anomalous situation and that open ocean bacteria can withstand nutrient deprivation for long periods of time and still retain the capacity for active metabolism, if the nutrients become available.

Starvation-survival has been defined as "the process of survival in the absence of energy-yielding substrates" (13). Marine bacteria isolated from open ocean environments are ideal for starvation-survival studies because their natural environment is exceedingly low in organic carbon (11), and they must often move with water masses for many years (18). Starvation-survival is important in ecology because it is a mechanism for the survival of a species in that the genome will persist and be able to express itself when the environmental conditions become favorable.

Ant-300, a marine psychrophilic vibrio, has been extensively studied for starvation-survival (15–18). When Ant-300 undergoes starvation-survival, the following takes place. (i) There is an initial large increase in the number of viable cells, followed by a decline until a constant level results, (ii) the cells decrease in size, changing from rods to coccobacilli, (iii) the endogenous respiration drops to less than 1% in 7 days, and (iv) they retain the ability to quickly metabolize substrates.

To determine whether Ant-300 is unique in its starvation-survival process, starvation-survival studies were conducted on 16 open ocean isolates.

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MATERIALS AND METHODS

Organisms. The 16 cultures used in this study were isolated by J. A. Baross during the NORPAX cruise (May to July, 1980) from nutrient-poor open ocean waters and depths 50 m or greater. Initial isolation was made on agar media low in or lacking added organic carbon (5°C incubation). Each culture was transferred just once to a low-level complex medium before these studies were conducted, to minimize the changes in characteristics known to occur in laboratory-maintained cultures. Due to the random sampling of the cultures and the wide variety of sizes and shapes displayed by the cells upon initial isolation, it was felt that an unbiased selection of the isolates had been obtained. Ant-300 was used in this study for comparative purposes. The source, substrate, and location from which the organisms were isolated is given in Table 1.

Media. The 16 cultures were isolated on media listed in Table 1. SLX medium is composed of 0.12 g of yeast extract (Difco Laboratories); 0.23 g of trypticase; 0.03 g of sodium citrate; 0.03 g of L-glutamic acid; 0.05 g of $NaNO_3$; 0.005 g of $FeSO_4$; 28 g of Rila Marine Mix; and 1 liter of distilled water. The pH was adjusted to 7.8. When solid medium was needed, 12 g of agar (Difco) was added per liter. Sulfur plus metals and formate plus NH_4^+ agar media contained an extensive salts-metal base with added sulfur and formate. The later media constituents are described by Baross et al. (1) and are considered to be chemoautotrophic and low organic media, respectively (Baross, personal communication).

Growth and starvation-survival procedure. All cultures were grown in SLX broth at 5°C in a Psychro-

TABLE 1. NORPAX cruise isolate characteristics

Isolate	Source	Station location	Pigmentation	Growth at 21°C	Isolation medium
N1	50 m water	Oregon coast (W8006)[a]	Tan	+	Sulfur + metals
N2	4,000 m water	Blanco hole (W8006)	Tan	+	SLX
N4	4,300 m squid pen	Station N9[b]		+	SLX
N5	4,000 m water	Blanco hole (W8006)	Yellow	+	SLX
N6	4,000 m water	Blanco hole (W8006)	Tan	+	SLX
N7	4,300 m water	Blanco hole (W8006)		+	Sulfur + metals
N8	1,700 m squid pen	Station N9		+	SLX
N9	1,700 m squid pen	Station N9		+	SLX
N10	3,800 m sediment trap	Station N8[c]		+	Formate + NH_4^+
N11	3,800 m sediment trap	Station N8		+	Formate + NH_4^+
N12	3,800 m sediment trap	Station N8		+	Formate + NH_4^+
N13	3,800 m sediment trap	Station N8		+	Formate + NH_4^+
N14	1,700 m squid pen	Station N8	Tan	−	Sulfur + metals
N15	1,700 m squid pen	Station N8		+	Sulfur + metals
N16	4,300 m crab shell	Station N9	Yellow	+	SLX
N17	4,300 m crab shell	Station N9		+	SLX

[a] W8006 = Cruise number of extension to NOPPAX cruise.
[b] N9 = Station location Latitude 0° 43.3'S, Longitude 153° 04.5'W, depth 4,865 m.
[c] N8 = Station location Latitude 0° 0.28'S, Longitude 152° 01.3'W, depth 4,312 m.

therm Gyrotory shaker, model G2 (New Brunswick Scientific Co.). Mid- to late-log phase cells were harvested by centrifugation at 4,080 × *g* in a Sorvall RC-2 refrigerated centrifuge for 15 min at 5°C. The cellular pellet was washed with cold buffered salts medium (SM) which contained 21.6 g of NaCl; 0.66 g of KCl; 6.3 g of $MgSO_4 \cdot 7H_2O$; 4.7 g of $MgCl_2 \cdot 6H_2O$; 1.2 g of Tris; and 1 liter of distilled water. SM was adjusted to pH 7.8. The washing and harvesting procedure was repeated twice. The starvation suspension in SM contained 10^7 to 10^8 cells per ml. The cells in this suspension were starved at 5°C. The suspension container was placed in a Psychrotherm reciprocating shaker (New Brunswick) at 100 rpm. All procedures were carried out under aseptic conditions.

Viability determinations. Viability was determined by the spread plate technique, using duplicate samples and cold SLX agar plates. Incubation was either at 5 or 21°C for up to 2 weeks.

Epifluorescence microscopy. Starved cells were diluted, fixed with Formalin, filtered onto 0.20-μm Nuclepore filters prestained with Irgalin black, and stained with 0.01% acridine orange (7, 22). After 10 min of staining, filters were washed twice with distilled water. All solutions were either filter sterilized or filtered before heat sterilization. The prepared filters were observed for total cell counts and shape changes. At least 10 fields from each of the replicate samples were counted.

Electron microscopy. Samples for morphological studies were prepared by centrifuging a mixture of 1.5 ml of cell suspension and 0.15 ml of 10% glutaradehyde (Tousimis) in a Beckman Microfuge B for 4 min, followed by a distilled water rinse and application of 0.1 ml concentrated cells in water to a plastic-coated copper grid. Excess water was blotted off, and cells were chromium shadowed in a vacuum evaporator (Varian VE 10) and viewed with a Philips EM 300 microscope. Representative cells were photographed and used for size determinations, presence of flagella, and shape.

Uptake and respiration studies. A 10-ml sample of the starvation suspension (see above) was diluted 1:10 in sterile cold SM containing 2 μM and 20 μM of phosphate and nitrate, respectively, and was incubated in the presence of ^{14}C-labeled substrates in 25-ml serum bottles with caps and buckets at 5°C for various lengths of time. The phosphate and nitrate were added in concentrations typically found in open ocean waters. The ^{14}C-labeled substrates were added to a final concentration of 0.004 μCi of D-[U-^{14}C]glucose per ml (329 μCi/μmol) and 0.005 μCi of L-[U-^{14}C]glutamic acid per ml (285 μCi/μmol). Both radioactive compounds were obtained from New England Nuclear Corp. The reaction was terminated by the addition of 0.2 ml of 5 N H_2SO_4 followed by shaking at 20°C for 1 h. The heterotrophic activity method was employed to determine the respired radioactive CO_2 and radioactivity incorporated into cells (2, 4–6, 21). All samples were counted in a Beckman LS-100C liquid scintillation counter for 10 min.

Percent respiration was calculated from the following formula: cpm $^{14}CO_2$/cpm $^{14}CO_2$ + cpm cellular incorporation × 100 = percent respiration.

RESULTS

The first pattern of starvation-survival was identical to that shown by Ant-300 (17). This pattern was displayed by seven (N5, N6, N8, N10, N15, N16, N17) of the NORPAX cruise isolates (Fig. 1A). Four of the isolates (N4, N7, N11, N13) displayed a second pattern of rapid death until a constant viability was attained (Fig. 1B). This constant viable count was approximately 10^4 and 10^8 per ml for N4 and N11, respectively. The third pattern (Fig. 1C) was displayed by five isolates (N1, N2, N9, N12, N14). The viable cell counts continued to increase during the first 4 to 16 weeks of starva-

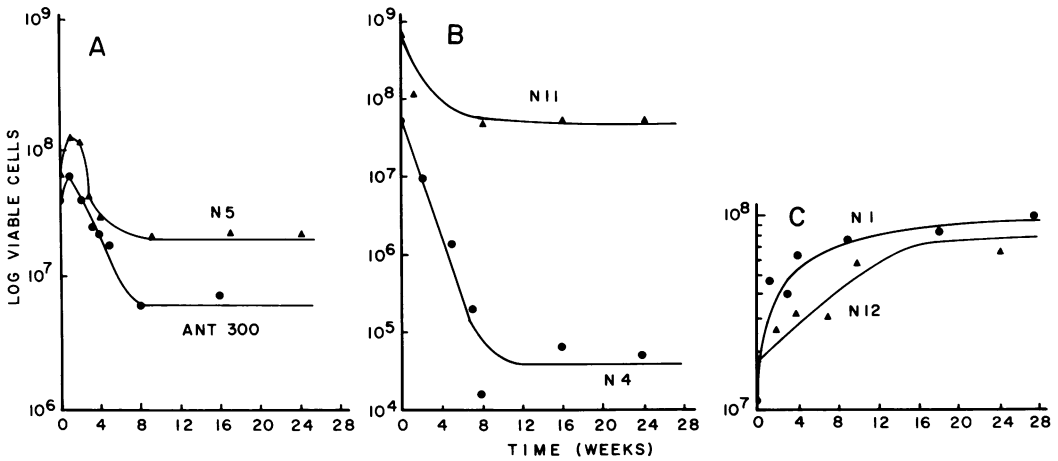

FIG. 1. Viability patterns of 17 open ocean isolates during nutrient starvation. The pattern of Ant-300 and 7 of the 16 isolates showed initial cell fragmentation followed by decrease in cell viability (A); the pattern of initial rapid cell death was seen with 4 of the 16 isolates (B); and the unusual pattern of initial viable cell increase was seen in 5 of the 16 isolates (C).

tion-survival. In N1 and N12, the viable counts reached nearly 10^8 cells per ml.

Epifluorescent cell counts showed that no isolates lysed during the 6-month starvation-survival period. Only N4 became non-viable and completely lysed, but only after 6 months of starvation. During the starvation-survival process, all isolates became smaller, and morphological changes in few of the isolates were noted (Table 2). In a few cases, flagellation was lost after 8 months of starvation. Fig. 2 illustrates some of the changes in morphology that took place during starvation-survival. Some isolates retained their original shape. Many rods became coccobacillary forms. N13, a vibrio during log growth, became a spirillum during starvation, and prolonged starvation for 8 months resulted in a coccoid form (Table 2).

To determine whether energy from respiration was required before starved cells could incorpo-

TABLE 2. Morphological changes during starvation, flagellation, and gram reaction of open ocean isolates

Isolate no.	Morphological changes after starvation[a]				Flagellation		Gram reaction
	Log phase cells	1 Mo	2 Mo	7–9 Mo	Grown log phase cells	Starved (8 mo)	Log phase cells
N1	R	R	R	CB	+	+	+
N2	R	R	C	CB	+	+	−
N4	R	C	C	Lysed	ND	ND	−
N5	R	R	R	R	+	+	−
N6	R	R	R	R	+	+	+
N7	v	S	C	ND	ND	ND	−
N8	v	v	R	CB	+	+	−
N9	S	R	R	R	+	+	−
N10	v	R	R	R	+	+	−
N11	S	S	S	CB	+	+	v
N12	v	R	R	R	+	+	v
N13	v	S	S	C	+	−	−
N14	R	C	C	C	+	+	−
N15	R	R	R	R	+	−	v
N16	R	ND	R	R	−	−	+
N17	S	S	S	ND	ND	ND	−
Ant-300	R	R	CB	CB	+	+	−

[a] Cell shape changes were determined by electron or epifluorescence microscopy. Abbreviations: R, rod shape; V, vibrio shape; C, coccoid shape; S, spirilla shape; CB, coccobacilli shape; v, Gram variable; ND, not determined.

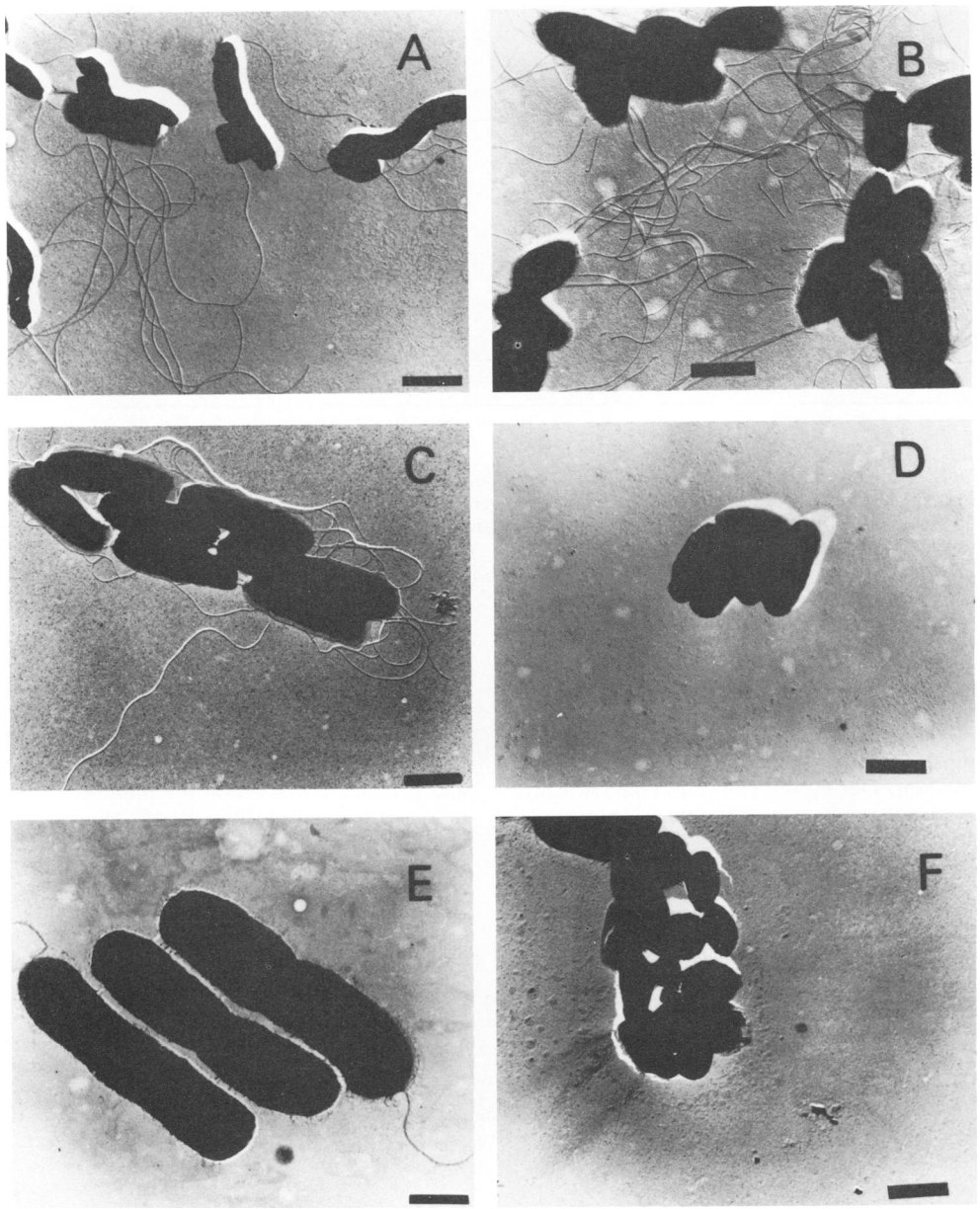

FIG. 2. Electron microscopy of cells from growth and starvation conditions. Isolate N10 grown (A) and starved 8 months (B), isolate N15 grown (C) and starved 8 months (D), and Ant-300 grown (E) and starved 4 months (F). Bar, 1 μm.

rate organic substrate, ^{14}C-labeled substrates were employed (Fig. 3 and 4). Four-month-starved Ant-300 cells responded immediately to the presence of glutamate and glucose (Fig. 2A, 2B), and the incorporation of substrates into cellular material and CO_2 production was linear. An 85% respiration of [^{14}C]glutamate was seen (Fig. 2A) compared with 5% respiration with [^{14}C]glucose (Fig. 2B). However, when replotted with comparable ordinates, the values for incorporation of [^{14}C]glucose and [^{14}C]glutamate were very similar (Fig. 2C). During starvation, the percent respiration with glutamic acid did not significantly change, although the rate

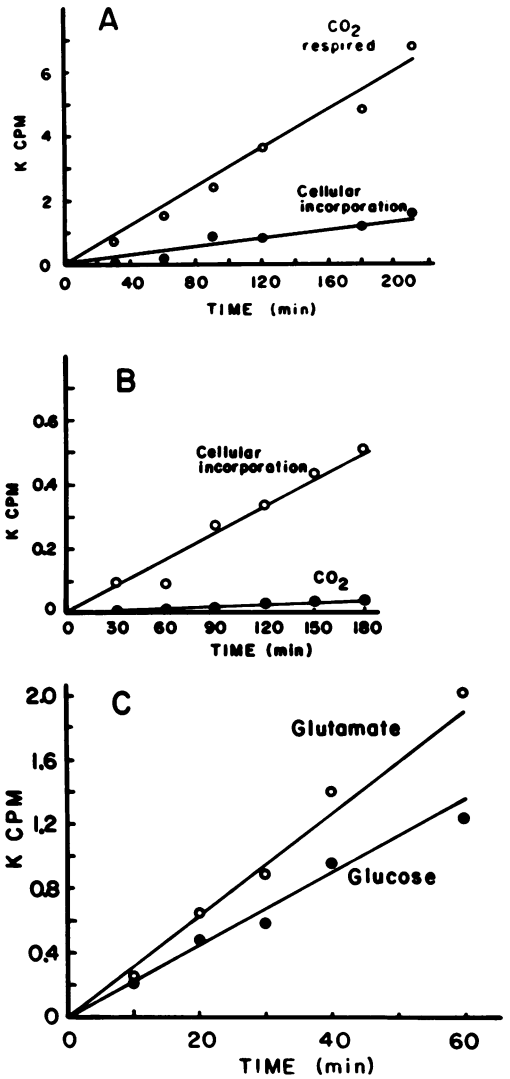

FIG. 3. Uptake and utilization of ^{14}C-labeled substrates by cells of Ant-300 starved for 3.5 months. (A) $^{14}CO_2$ (○) and cellular incorporation (●) of [^{14}C]glutamic acid. (B) $^{14}CO_2$ (○) and cellular incorporation (●) of [^{14}C]glucose. (C) A comparison of cellular incorporation of [^{14}C]glutamic acid (○) and [^{14}C]glucose (●). Each point is the average of triplicate samples.

was decreased with starvation time (M. A. Glick, M.S. thesis, Oregon State University, Corvallis, 1980).

Isolate N5, with the same starvation-survival viability pattern as Ant-300, also displayed an immediate and linear incorporation and respiration of [^{14}C]glutamate (Fig. 4A). Isolate N11, which displayed a pattern of rapid cell death upon starvation (Fig. 1B), also displayed a rapid linear cellular incorporation and respiration of [^{14}C]glutamate (Fig. 4B). Although isolate N1 (Fig. 1C) respired [^{14}C]glutamate linearly, the cellular incorporation of the same substrate was extremely rapid (Fig. 4D).

DISCUSSION

All isolates demonstrated survival under starvation conditions. Miniaturization of cells appeared to be a common phenomenon during starvation-survival, and some isolates lost their flagella. This reduction in size of the cell may be quite significant (Fig. 2). The miniaturization of cells increased the surface-to-volume ratio of these cells. A larger surface/volume ratio may aid cells in obtaining substrates from a nutrient-poor environment (13) and in the starvation-survival process (10, 17).

Some ultramicrocells (as defined by Torrella and Morita [19]) in the marine environment are, more than likely, formed by the lack of specific nutrients such as vitamins and essential amino acids, since ultramicrocells can be found even in near shore environments where organic carbon is more abundant. In fact, many researchers have reported that ultramicrocells become larger cells when placed on media (8, 9, 19), whereas Torrella and Morita (19) could not demonstrate a size increase of all ultramicrocells even under these conditions. Watson et al. (20) reported that approximately half of the organisms observed during a cruise were ultramicrocells.

All of the tested, starved cell cultures demonstrated the ability to produce $^{14}CO_2$ from ^{14}C-labeled substrates. This is a good indication that the metabolic systems necessary to produce energy in starved cells remained intact. The same can be said for the mechanism for incorporation of ^{14}C-substrates into cellular material. Starved cells have the ability to capture substrates (12).

In conclusion, 16 open ocean marine isolates and Ant-300 demonstrated the ability to withstand starvation conditions, became smaller, and retained the ability to metabolize small quantities of [^{14}C]glutamic acid in a manner typical of natural populations (3, 4, 14). All isolates used in this study were picked at random from various isolates obtained by J. A. Baross on the NORPAX cruise, and all isolates displayed one of three starvation-survival patterns. This study is a good indication that most, if not all, species of marine bacteria have a mechanism to survive long periods of time without an external energy source. The starvation-survival process helps to ensure that a bacterial species can survive long periods of energy deprivation and that the surviving starved cells can express themselves when conditions become favorable again.

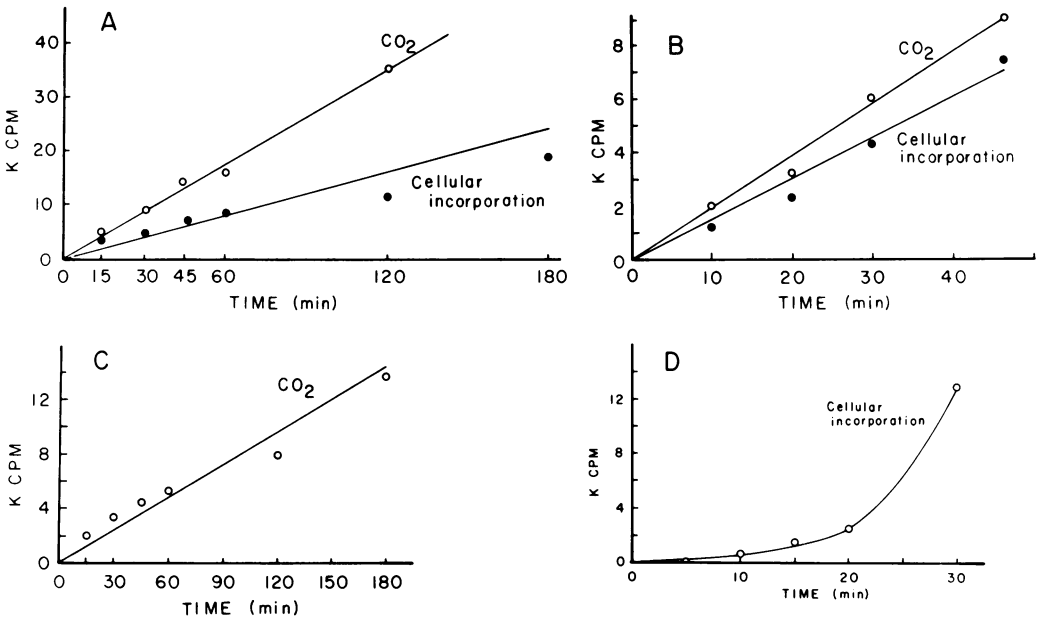

FIG. 4. Respiration and cellular incorporation of [^{14}C]glutamic acid by representatives of each viability pattern. (A) $^{14}CO_2$ and cellular incorporation by N5 cells starved for 8 months. (B) $^{14}CO_2$ and cellular incorporation by N11 cells starved for 8 months. (C) $^{14}CO_2$ evolved by N1 cells starved for 9 months. (D) Cellular incorporation by N1 cells starved for 9 months. The viable cell count was 2.5×10^5 cells per ml for all experiments. Each point is the average of triplicate samples.

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